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Evaluation of In Vitro Enzymatic Degradation of Various Thiomers and Cross-Linked Thiomers

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ABSTRACT The aim of this study was to examine the biodegradability of thiomers and cross-linked thiomers in comparison with unmodified polymers. Disulfide-cross-linked conjugates were prepared by air oxidation at room temperature. Thiomers were investigated by viscosity measurements and spectrophotometric assays. The influence of different factors on the hydrolysis rate, such as the degree of modification of thiomers, structure of the conjugates, pH value of the reaction medium, and the impact of the process of cross-linking were evaluated. Due to the modification, thiolated chitosans degraded 12.9–24.7% less than unmodified chitosan in the framework of viscosity measurements. In addition, the hydrolysis degree of thiolated alginates and modified carboxymethylcelluloses was 25.6–32.4% and 18.4–27.0% lower, respectively, in comparison to the corresponding unmodified polymers. Conjugates with higher coupling rate of thiol groups were degraded even more slowly. Moreover, the cross-linking process via disulfide bonds additionally reduced the rate of thiomer degradation. The range of degradation rates achieved in vitro could be modified by alterations of the contents of thiol and disulfide groups, as well as by suitable design of the polymer structure and ligands used.

These results represent helpful basic information for the development of mucoadhesive drug delivery systems, implantable delivery systems and tissue engineering constructs.

KEYWORDS Thiolated chitosans, Thiolated alginates, Thiolated carboxymethylcelluloses, Thiomers, Enzymatic degradation, Lysozyme, Alginate lyase, Cellulase, Viscosity measurements

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INTRODUCTION

The new thiomer technology offers an intelligent approach for the improvement of mucoadhesion by using thiolated polymers, designated as thiomers (Bernkop-Schnürch et al., 2004). The thio-ligands of thiomers are believed to interact with cysteine-rich subdomains of mucus glycoproteins via covalent disulfide bonds. Apart from their improved mucoadhesive properties, thiomers also exhibit permeation-enhancing, swelling behavior, strong cohesive

and pancreatic enzyme inhibitory properties (Bernkop-Schnürch & Steininger, 2000; Kast et al., 2003). Among all the synthesized and evaluated thiolated polymers, modified natural polymers represent an area of active research because they are readily available, potentially biodegradable and compatible with the physiological environment. Such examples are thiolated chitosans, alginates and carboxymethylcelluloses (Bernkop-Schnürch et al., 2004). The chemical modification of polymers can enhance or reduce the rate of biodegradation (Lee et al., 1995). The biodegradability of component materials is critical when designing a drug delivery device. The erosion process occurs either in bulk wherein the matrix degrades uniformly, or at the polymers surface whereby release rates are related to the polymer surface area. Therefore, varying each of these factors allows researchers to adjust the rate of matrix degradation and, subsequently, control the rate of drug delivery. Based on this approach, various polysaccharides (chitosan, pectin) have been investigated for colon-specific drug release. The matrices of polysaccharides are assumed to remain intact in the physiological environment of the stomach and small intestine, but once they reach the colon, they are acted upon by the bacterial polysaccharidases, leading to the degradation of matrices. Many studies have shown that chitosans degrade in vivo mainly through their susceptibility to enzymatic hydrolysis mediated by lysozyme (Vårum et al., 1996; Kristiansen et al., 1998; Kast & Bernkop-Schnürch 2001).

The alginate-cysteine conjugate represents the first anionic biodegradable polymer, which has been chemically modified by the covalent attachment of thiol groups (Bernkop-Schnürch et al., 2001a,b). Most of the lyases studied so far, prefer mannuronic (M) blocks (Wong et al., 2000).

The cellulose thiolated polymers have been synthesized and characterized in vitro, based on water-soluble, biocompatible carboxymethylcellulose (Bernkop-Schnürch Thaler, 2000; Bernkop-Schnürch et al., 2001a,b). Biodegradability of cellulose derivatives can be controlled by hydrolysis and cytocompatible enzyme (cellulase) action, with glucose as a final product (Entcheva et al., 2004).

In order to assess the stability of thiomers under hydrolytic conditions encountered in vivo, the polymers were exposed to various enzymes and examined for degradation in comparison with unmodified polymers. The objective of our study was also to investigate the influence of certain factors on biodegradation, such as the degree of modification of the polymer, the nature of the thiomer ligand, the molecular weight of polymer and pH of the medium for hydrolysis.

MATERIALS AND METHODS

Materials

Chitosan (medium molecular mass: 400 kDa; degree of deacetylation: 83–85%) was obtained from Fluka Chemie (Buchs, Switzerland). Lysozyme from white of a chicken egg (EC 3.2.1.17, 150000 U/mg) was purchased from Serva Electrophoresis GmbH. Sodium salt of Alginic acid with low (~250 cP) and medium viscosity (~3500 cP), alginate lyase from *Flavobacterium* sp. (EC 4.2.2.3, 10000 U/g), micrococcus lysodeikticus lyophilized cells, cellulase from *Aspergillus niger* (EC 3.2.1.4, 0.3 U/mg), and glucose (HK) assay kit were all purchased from Sigma-Aldrich (St. Louis, MO). Sodium carboxymethylcellulose with average molecular weight of 1000 kDa was procured from Kwizda, Vienna, Austria.

Synthesis of Thiomers

Synthesis of thiolated chitosans

Chitosan thioglycolic acid conjugate (Ch-TGA) was synthesized by a method described previously (Kast et al., 2003). The coupling reaction was mediated by carbodiimide (EDAC) in two final concentrations of 50 or 100 mM in order to obtain conjugates with contents of different thiol groups.

Chitosan-glutathione (Ch-GSH), chitosan-4-thiobuthylamidine (Ch-TBA) and chitosan-thioethylamidine (Ch-TEA) conjugates were synthesized according to methods described earlier (Kafedjiiski et al., 2005a,b; Bernkop-Schnürch et al., 2003).

Synthesis of thiolated alginates

The alginate-cysteine (alginate-Cys) conjugates (low and medium viscosity) used in this study were synthesized according to the method of Bernkop-Schnürch et al. (2001a).

Synthesis of thiolated carboxymethylcelulloses

Carboxymethylcellulose-cysteine (CMC-Cys) and carboxymethylcellulose-cysteamine (CMC-cysteamine) conjugates were prepared according to methods described previously (Bernkop-Schnürch & Steininger, 2000; Bernkop-Schnürch et al., 2001b).

Cross-linking of Thiomers

Disulfide-cross-linked conjugates were prepared by dissolving thiomers in 100 mM acetate buffer, pH 6.0, in a final concentration of 1% (w/v). Afterwards, they were oxidised in open air at room temperature for 1 day.

Determination of Thiol Groups and Disulfide Bonds Within the Polymers

The amount of thiol groups immobilized on the conjugates was determined spectrophotometrically using Ellman's reagent quantifying free thiol groups as described previously (Kafedjiiski et al., 2005b).

Disulfide bond content was determined after reduction with NaBH4 and addition of Ellman's reagent as described by Habeeb (1973).

In Vitro Enzymatic Degradation

Thiolated chitosans

Viscosity measurement of thiolated chitosans was done by preparing 1% (w/v) polymer solution (9.0 mL) in 100 mM acetate buffer at pH 5.0–7.0 at 37°C. Then, 0.5 mg/mL lysozyme solution (1 mL) in a buffer was added. At predetermined time points, the viscosity of 1 mL aliquots of the mixtures was measured at 37 ± 0.5°C with a Rheolab MC 1 (Paar Physica GmbH, Stuttgart, Germany) cone-plate rheometer. Indicated viscosity was determined at a shear rate of 10/s. The change of viscosity at time t was normalized by the viscosity at time zero. The percent viscosity loss was calculated from the following equation:

$$\text{Percent } \eta_1 = (\eta_0 - \eta_t/\eta_0)100$$

where,

percent η_1 = percent viscosity lost after time t;

η_t = final viscosity at time t;

η_0 = initial viscosity of solution.

The effect of thiol ligands on the enzyme activity of lysozyme was determined by the turbidimetric lysozyme assay (Shugar, 1952). In a typical experiment, decreasing amounts of thiol ligand (GSH) were dissolved in 500 µL of 66 mM potassium phosphate buffer (pH 6.24) in final concentrations of 6.6, 3.25, 1.625, 0.812, and 0.406 mM. To these solutions, 200 units of lysozyme were added. After incubation for 60 min at 37°C, aliquots of 100 µL were transferred to 2.5 mL of the substrate *Micrococcus lysodeikticus* cell suspension (0.015%, w/v) in the same buffer. For control, the thiol solution was replaced with buffer. The decrease in absorbance was immediately measured at 450 nm for 5 min at 25°C (UV spectrophotometer-1202, Shimadzu, Japan). The activity was calculated from the slope of the linear region of the A_{450nm}/time curve, assuming that one unit of enzyme activity will reduce the A_{450nm} by 0.001/min under the condition used. Effect of thiols on the enzyme activity was calculated according to the equation

$$A\% = (\Delta A_{thiol}/\text{min} - \Delta A_{blank}/\text{min})/(\Delta A_{control}/\text{min} - \Delta A_{blank}/\text{min})$$

Thiolated alginates

In order to measure viscosity of thiolated alginates, to 9.0 mL of 1% (w/v) polymer solution in demineralized water, 0.5 mg/mL alginate lyase solution (1 mL) was added at pH 6.8 and 25°C. At predetermined time points, the viscosity of 1 mL aliquots of the mixtures was measured at 25 ± 0.5°C as described above.

Spectrophotometric measurement was based on the methodology adopted by Iwamoto et al. (Iwamoto et al., 2001), with marked absorbance recorded at 235 nm of unsaturated urinates produced by the enzymatic degradation of alginate.

Thiolated carboxymethylcelulloses

Viscosity was measured by adding 1 mg/mL cellulase solution (1 mL) to 9 mL of 1% (w/v) polymer solution in 50 mM acetate buffer at pH 5.0 and 25°C. At predetermined time points, the viscosity of 1 mL aliquots of the mixtures was measured at 25 ± 0.5°C as described above.

In glucose assay the rate of hydrolysis was monitored by the enzymatic determination of glucose

liberated from CMC derivatives under the conditions of viscosity measurements. The assay was carried out according to the method of Bondar & Mead (1974) using Sigma protocol for glucose kit. Briefly, 100 μL aliquots were taken from the reaction mixture and incubated with 0.5 mL of glucose reagent for 15 min at room temperature. The absorbance was measured at 340 nm at 25°C. The increase in absorbance is directly proportional to glucose concentration:

$$\text{mg glucose / mL} = (\Delta A)(TV)(F)(0.029)/(SV)$$

where,
ΔA is the absorbance;
TV = total assay volume (mL);
F = dilution factor;
SV = sample volume (mL).

Statistical Data Analysis

Statistical data analyses were performed using the student's *t*-test with $p < 0.05$ as the minimal level of significance. Statistical comparisons (*p*-values) were made using two-sided Student's unpaired *t*-test.

RESULTS AND DISCUSSION

Synthesis and Basic Characterization of Thiomers

The presumptive chemical substructures and characteristics of the tested thiomers are presented in Fig. 1a,b and Table 1. The formation of disulfide cross-linked thiomers was achieved by air oxidation of thiomer solutions under mild conditions at room temperature (Fig. 1a). For example, the disulfide bonds in Ch-GSH conjugate increased from 137.9 – 214.1 μM/g.

In Vitro Enzymatic Degradation

Thiolated chitosans

One of the most sensitive methods to study the kinetics of the enzymatic degradation of chitosans is viscosimetry (Lee et al., 1995). Lysozyme is active over a broad pH range of 5.2–8.0 (Davies et al., 1969). Accordingly, the influence of pH values on the degradability of thiolated chitosans, investigated in

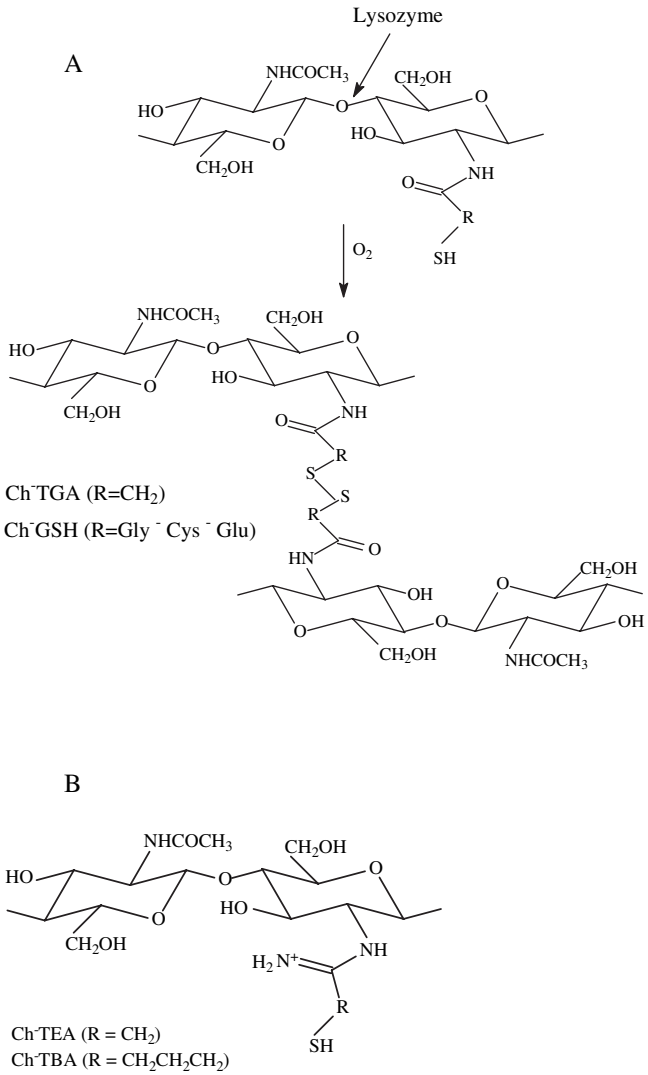


FIGURE 1 **(a) Presumptive Chemical Substructures of Amide Thiolated Chitosans and Cross-linked Amide Thiolated Chitosans. (b) Presumptive Chemical Substructures of Amidine Thiolated Chitosans.**

the case of Ch-GSH conjugate, is presented in Fig. 2. The viscosity loss, which corresponded to the hydrolysis of the initial chitosan in the linear part of dependence up to 60 min, was 39%. Chitosan without lysozyme remained almost stable under these conditions. All samples of Ch-GSH conjugate exhibited a slower degradation compared with unmodified polymer. The loss of viscosity of Ch-GSH samples after 60 min at pH 7, 6, and 5 were 19.1, 23.6 and 25.7%, respectively. The difference between degradability at pH 5 and 6 was of no statistical significance. In contrast, when the values were compared with the results obtained at pH 7, a statistically significant difference was recorded (two-sample *t*-test, $p < 0.03$). This observation might be explained by the lysozyme inactivation

TABLE 1 **Characterization of the Thiomers Used with Various Amounts of Immobilized Free Thiol and Disulfide Groups on the Polymeric Backbone. Indicated Values are Means of Three Measurements (n = 3, ± SD)**

Thiomers	Molecular mass (kDa)	Thiol groups (μmol/g)	Disulfide groups (μmol/g)
Chitosan-TGA 1 (100 mM EDAC)	400	252.6 ± 18.1	91.3 ± 16.8
Chitosan-TGA 2 (50 mM EDAC)	400	94.2 ± 10.8	41.5 ± 8.7
Chitosan-TBA	400	309.5 ± 62.1	136.2 ± 24.3
Chitosan-TEA	400	269.8 ± 29.0	116.0 ± 18.5
Chitosan-GSH	400	265.5 ± 7.8	137.9 ± 28.2
Chitosan-GSH (cross-linked)	400	189.3 ± 28.2	214.1 ± 36.2
Alginate-Cys (low viscosity)	–	234.3 ± 23.6	87.3 ± 14.6
Alginate-Cys (medium viscosity)	–	276.1 ± 33.8	126.4 ± 21.8
Carboxymethylcellulose-Cys	1000	325.7 ± 20.5	168.7 ± 31.4
Carboxymethylcellulose-Cysteamine	1000	106.3 ± 13.5	42.4 ± 8.9

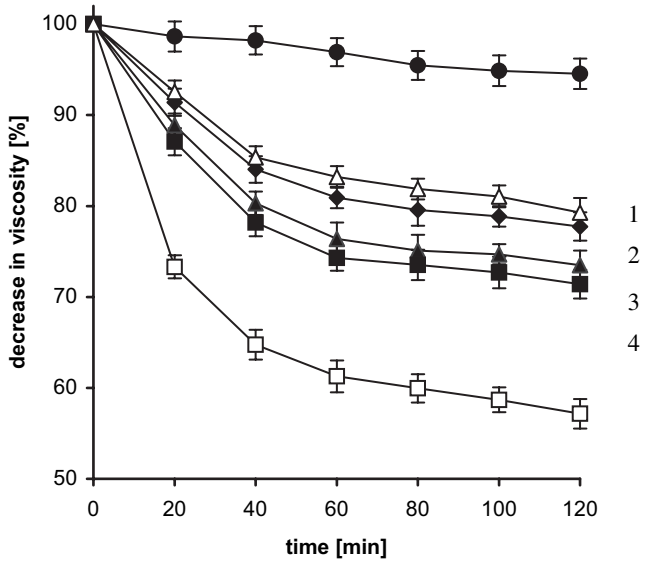


FIGURE 2 **Effect of the pH and Cross-linking of Ch-GSH on the Decrease in Viscosity of Ch-GSH Solutions by Enzymatic Degradation. Lines Represent Chitosan Without Lysozyme at pH 6 (●), Ch-GSH pH 7 (✦), Ch-GSH pH 6 (▲), Ch-GSH pH 5 (■), Ch-GSH Cross at pH 6 (△) and Chitosan at pH 6 (□). Indicated Values are Means (±SD) of at Least Three Experiments;[3, 4], differ from[2], p < 0.03 and[1], differs from[3], p < 0.01. (Superscripted numbers demonstrate statistical differences between points.)**

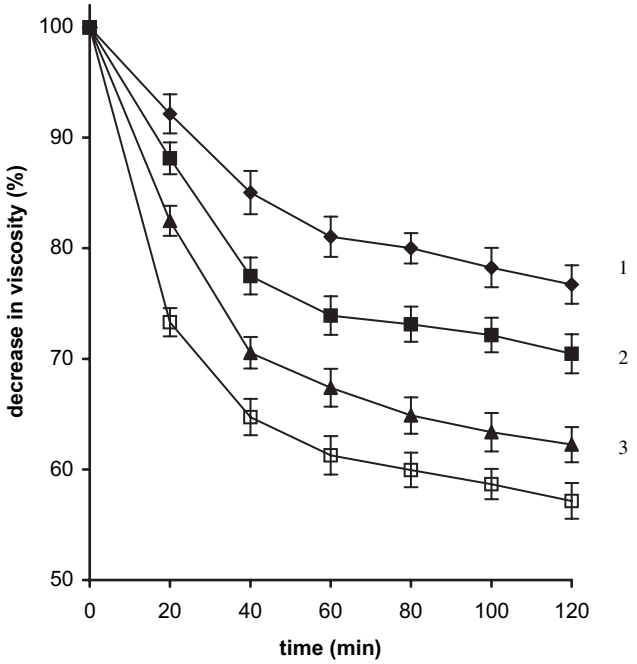


FIGURE 3 **Effect of the Degree of Modification and Cross-linking on the Decrease in Viscosity of Ch-TGA Solutions by Enzymatic Degradation at pH 6.0. Lines Represent Ch-TGA 1 Cross (✦), Ch-TGA 1 (■), Ch-TGA 2 (▲) and Chitosan (□). Indicated Values are Means (±SD) of at Least Three Experiments;[1,2,3], Differ from Chitosan, p < 0.02. (Superscripted numbers demonstrate statistical differences between points.)**

effect of thiolated chitosans. The reduced enzymatic activity of lysozyme might be a result of interactions between thiol moieties of the conjugate and disulfide bonds of lysozyme. The higher pH 7 was beneficial and influenced the reductive cleavage of disulfide bridges in lysozyme.

Effect of cross-linking of thiomers was obvious as in vitro degradation of cross-linking thiomers is shown for Ch-GSH in Fig. 2 and Ch-TGA conjugates in Fig. 3. Both, the thiomer and cross-linked Ch-GSH conjugates at pH 6, exhibited slow degradation rates but there was a significant difference between their rates of degradation (two-sample t-test, $p < 0.01$). The rate of degradation of the cross-linked thiomers was reduced as a result of a cross-linking process via disulfide bonds. This effect revealed that the disulfide bonds comprising both inter- and intramolecular cross-links between conjugate molecules were stable and rendered the thiomer resistant to hydrolysis. Nevertheless, the introduction of disulfide bonds did not completely alter the access to the glycosidic linkages, which can be still recognized and hydrolysed by lysozyme.

Effect of the degree of chemical modification was studied and the action of lysozyme on Ch-TGA conjugates with different thiol groups contents was compared with unmodified chitosan (Fig. 3). In case of Ch-TGA conjugates, the loss of viscosity after 60 min occurred slowly as compared to unmodified chitosan; 32.6% loss was observed for Ch-TGA 2 and 26.1% for Ch-TGA 1, respectively. It was obvious that the lower degree of hydrolysis was achieved for thiomer bearing a higher thiol groups content.

Thiolated chitosans obtained in the present study can be classified in two groups, amide and amidine substructures, depending on the kind of the covalent attachment of ligands (Fig. 1a,b). The amidine substructure (Ch-TBA, Ch-TEA) bears a cationic character and, thus, additionally influences the ionic interactions between polymer and other substructures. Comparison of the results is presented in Fig. 4. Among all thiomers, amidine conjugates Ch-TBA and Ch-TEA exhibited least rate of viscosity loss of 14.3% and 19.7%, respectively within 60 min. The amide conjugates were more susceptible to degradation. The influence of amidine bond is more pronounced when the rates of biodegradation between Ch-TEA and Ch-GSH conjugates exhibiting thiol groups content in the same range are compared. The significant difference (two-sample *t*-test, $p < 0.03$) in the rate of this process between amidine conjugates Ch-TBA and Ch-TEA conjugates could be explained by the higher amount of thiol groups of Ch-TBA. In addition, the ligand of Ch-TBA is more hydrophobic compared with the Ch-TEA ligand, therefore, has a negative influence on the solubility of this thiomer and its biodegradability, respectively.

Reduction studies have revealed that the primary structure of egg white lysozyme is a single polypeptide chain of 129 amino acids. There are four pairs of cysteines forming disulfide bridges (-S-S-) in the molecular structure of lysozyme. The disulfide bridges play a major role in the formation of the secondary structure of lysozyme. The enzymatic activity of lysozyme is lost when more than two disulfide bridges are destroyed (White, 1982).

In reduction studies, we have chosen thiol compounds, appearing as ligands in thiolated chitosans. The variation of thiol concentration that thiomer achieved was relative to the concentration of thiol groups under the conditions of enzymatic degradation. To characterize the enzymatic activity of lysozyme after reduction, the activity towards the substrate *Micrococcus lysodeikticus* cell was measured as a function of the thiol concentration. In the case of glutathione, the ligand induced a concentration dependent partial inactivation of lysozyme activity in millimolar range (Fig. 5). The lysozyme activity versus GSH concentration curve showed a strong inactivation even at concentration of GSH below 3 mM. At 6.5 mM concentration there was loss of activity to the extent of 67%.

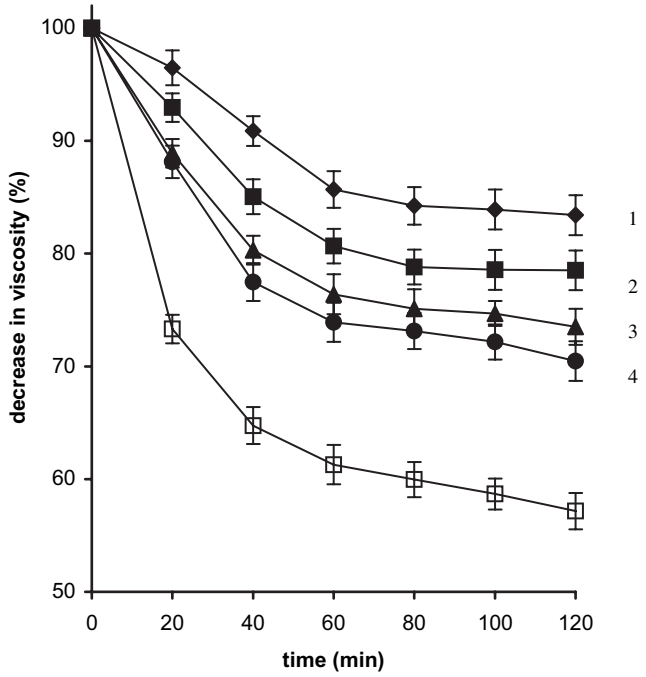


FIGURE 4 **Effect of Amidine and Amide Bonds of Ligands on the Decrease in Viscosity of Polymer Solutions by Enzymatic Degradation at pH 6.0. Lines Represent Ch-TBA (✦), Ch-TEA (■), Ch-GSH (▲), Ch-TGA 1 (●) and Chitosan (□). Indicated Values are Means (±SD) of at Least Three Experiments;[1], differs from[2], and[2], differs from[3], $p < 0.03$. (Superscripted numbers demonstrate statistical differences between points.)**

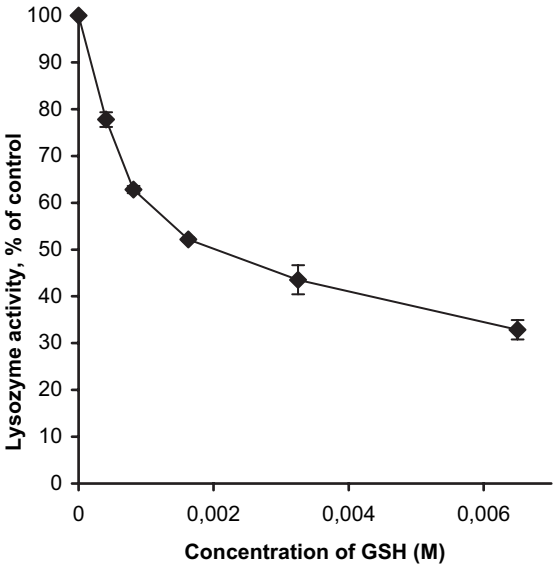


FIGURE 5 **Influence of Glutathione Concentration on the Lysozyme Activity. Indicated values are means (±SD) of at least three experiments.**

Hen egg white lysozyme bears well-known binding subsites, designated by the letters A-F and the glycosidic bond cleavage takes place between sites D and E. Chitosan is a linear polysaccharide of randomly distributed 2-acetamido-2-deoxy-β-D-glucopyranose (A-unit) and 2-amino-2-deoxy-β-D-glucopyranose (D-unit) units. The lysozyme catalysis studies haverevealed that the specificity of lysozyme has a preferential hydrolysis towards only 2 of 16 possible tetrad sequences, i.e., the sequences-A-A-A-A- and -A-A-A-D- are preferentially cleaved at the middle glycosidic bonds (Vårum et al., 1996). In thiolated chitosans, the modification of the structure covers only D-units. Therefore, the polymer remains a substrate susceptible to hydrolysis by lysozyme. Concerning amidine chitosans, it might be suggested that the additional positive charge of chitosan ligand will influence the reaction between substrate and acidic residues of lysozyme. Varum et al. found that local electrostatic interactions between the active site cleft of lysozyme and positively charged chitosans significantly contribute to binding interactions (Kristiansen et al., 1998). The process, which takes place in the thiomer system, can be described as a composite reaction for which the rate of disappearance of thiomer is governed by the rate constants relating to two simultaneous, "parallel" reactions (hydrolysis and reduction), leading to the formation of different products. The partial inactivation of lysozyme activity indicates the retention of part of disulfide bonds. The reactive form of thiol groups is the thiolate ion, where concentration depends on the pKa of the thiol groups of the polymer and pH of the reaction. Based on the chemical structure of the modified polymers, the pKa values of the thiol groups were determined to be in the range 8.5–9.5. The difference in pKa values of the thiol groups of the polymers also explains the results from the viscosimetry measurements.

Thiolated alginates

Alginate is comprised of mannuronic (M) and guluronic (G) sugars organized into blocks of M and G-residues. The blocks comprised of alternating residues and form space-filling hydrogels by ionic crosslinking with divalent cations such as calcium. The molecular weight of alginate regulates the chelation

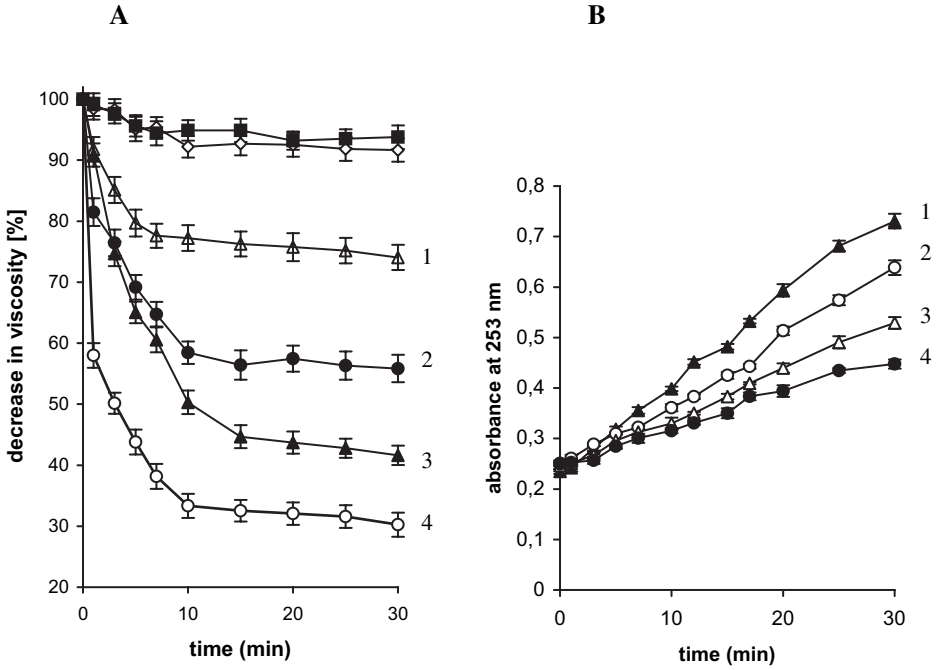


FIGURE 6 **(a) Comparison of the Degradation of Alginates and Alginate-Cys Conjugates by Alginate Lyase at pH 6.8. Lines Represent Sodium Alginate Medium Viscosity Without Lysozyme (■), Sodium Alginate Low Viscosity Without Lysozyme (◇), Alginate-Cys Low Viscosity (△), Alginate-Cys Medium Viscosity (●), Sodium Alginate Low Viscosity (▲) and Sodium Alginate Medium Viscosity (○). Indicated Values are Means (±SD) of at Least Three Experiments;[1], Differs from[3], $p < 0.00003$;[2], differs from[4], $p < 0.0001$. (b) Increase in Absorbance at 235 nm of Polymer Solutions (0.1%) by the Enzymatic Degradation at pH 6.3. Lines Represent Alginate-Cys Low Viscosity (△), Alginate-Cys Medium Viscosity (●), Sodium Alginate Low Viscosity (▲) and Sodium Alginate Medium Viscosity (○). Indicated Values are Means (±SD) of at Least Three Experiments;[1], differs from[3], $p < 0.00002$;[2], differs from[4], $p < 0.0001$. (Superscripted numbers demonstrate statistical differences between points.)**

of the cross-linking calcium and gel dissolution in vivo (Alsberg et al., 2003). Decreasing the size of the polymer chains increases the degradation rate in vivo.

The effect of alginate lyase on polymer viscosity is shown in Fig. 6A. With unmodified alginates, the viscosity dropped rapidly, whereas with thiolated alginates slow viscosity change was noted. The loss of viscosity of alginate-Cys (low viscosity) was 26% for 30 min, statistically different ($p < 0.00003$) from corresponding unmodified polymer (58.4%). The loss of viscosity of alginate-Cys (medium viscosity) was 44.2%, which was significantly less ($p < 0.0001$) when compared with unmodified alginate (69.8%). The results of a rapid initial decrease in viscosity can be interpreted as the cleavage of internal glycoside bonds (Nakada et al., 1967). The viscosimetric method proved to be sensitive and accurate, however, comparison of the results showed that polymers with different molecular mass were difficult to assess, since the half viscosity reduction times were found to be highly dependent on the initial viscosity of the polymer solution. The variation is known to depend on the molecular mass of the respective polymer (Alburn & Whitley, 1951). This explains the observed higher degradation rate of alginates with medium viscosity. In contrast, direct spectrophotometric measurements of unsaturated uronic acid products (Fig. 6b) demonstrated higher degradation rate of alginates with low viscosity. Kinetics of the process supports this view and it is in agreement with in vivo studies (Alsberg et al., 2003). Alginate lyase (EC 4.2.2.3) cleaves at the β-D-(1–4) mannuronic bond residues through a β-elimination reaction and produces unsaturated saccharides with a double bond between C4 and C5 sites in the nonreducing terminal sugar (Fig. 7) (Gacesa, 1992). The first step from the mechanism includes the removal of the negative charge on the carboxyl anion by a salt bridge (lysine may be the candidate residue). When the carboxyl group is modified with thiol ligand, as in thiolated alginate, it can be assumed that the first step of the salt bridge formation would not take place. Thus, it would lead to a decrease in the degree and the rate of degradation of the thiolated alginate.

Cellulase is a common name for a group of enzymes that attacks 1,4-β-glucosic bonds, thereby selectively breaking carboxymethylcellulose into smaller length chains down to its glucose repeat

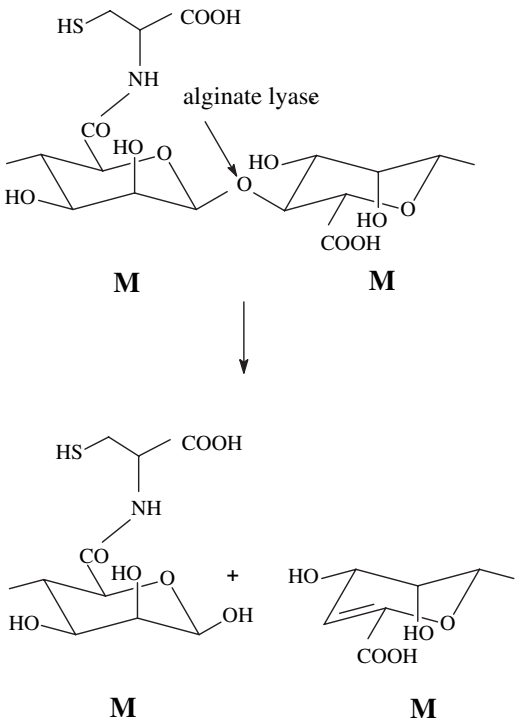
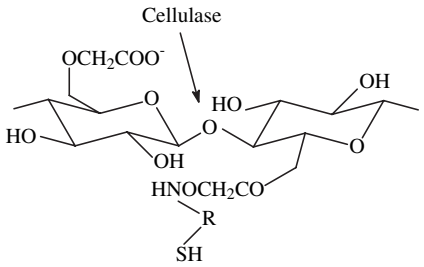


FIGURE 7 **Block Sites of Thiolated Alginate and Alginate Lyase reaction.**



CMC-Cys [R = $CH(COOH)CH_2$]
CMC-Cysteamine [R = CH_2CH_2]

FIGURE 8 **Presumptive Chemical Substructures of Thiolated Carboxymethylcelluloses.**

units (Fig. 8). The enzyme action was probed by two alternative methods; the loss of viscosity of polymer solution and assay of the release of glucose in solution (Fig. 9a,b). The hydrolysis degree obtained within 60 min by this enzyme dosage was high enough to allow comparison of the enzymatic degradability of different samples. The loss of viscosity of thiomers was 27.4% for CMC-Cys and 36% for CMC-Cysteamine, respectively, and it was significantly less than unmodified CMC (54.4%). The same dependence was obtained from the assay of released glucose. The two measurements of the hydrolysis process showed sufficient correlation

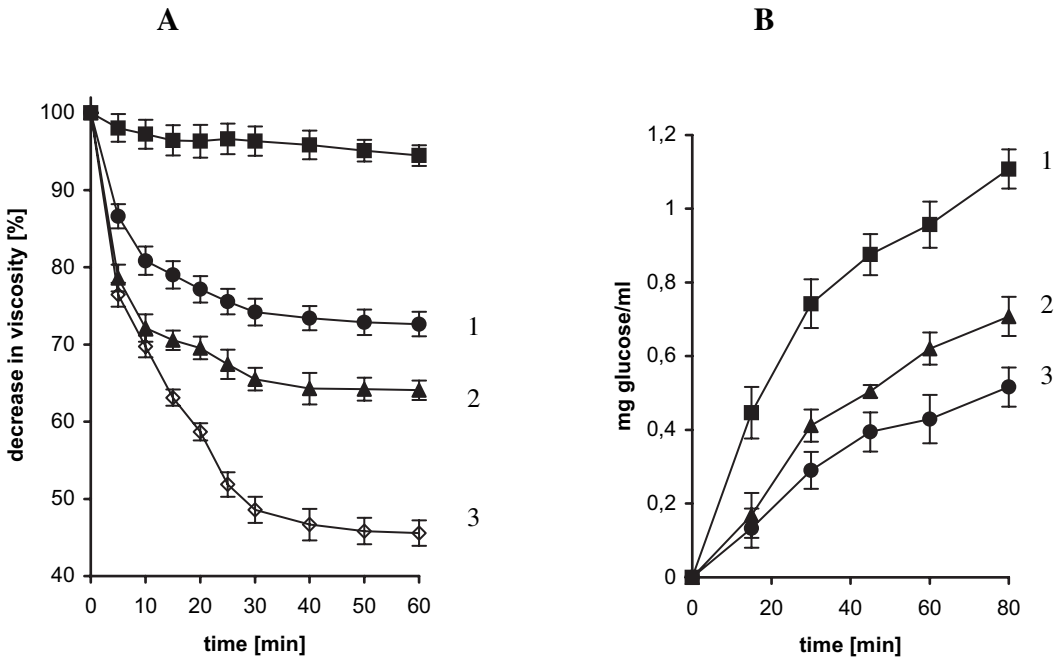


FIGURE 9 **(a) Comparison of the Degradation of Carboxymethylcellulose (CMC) and Thiolated CMC Conjugates by Cellulase at pH 5.0. Lines Represent CMC Without Cellulase (■), CMC-Cys (●), CMC-Cysteamine (▲), and CMC (◇). Indicated values are means (±SD) of at least three experiments;[1,2], differ from[3], $p < 0.0001$;[1], differs from[2], $p < 0.002$. (b) Release of Glucose from CMC Derivatives by Cellulase at pH 5.0. Lines Represent CMC (■), CMC-Cysteamine (▲) and CMC-Cysteine (●). Indicated Values are Means (±SD) of at Least Three Experiments;[2,3], differ from[1], $p < 0.0008$. (Superscripted numbers demonstrate differences between points.)**

($R^2 = 0.92$). The degree of thiol modification of carboxymethylcellulose affects hydrolysis in such a way that the more ligands immobilized on the substrate, the less efficient the hydrolysis. An explanation for this is that ligands act as steric hindrance for the enzyme. The residues with carboxylic side chain–(aspartate/glutamate), tryptophan, and cysteine-were shown to be responsible for the catalytic activity of cellulase from *Aspergillus niger* (Singh et al., 1990). Therefore, it might be suggested that an interaction between thiol groups of the conjugate and cysteine of cellulase takes place, thus, causing an inactivation of cellulase. The activity of the thiol groups of the ligands cysteamine and cysteine is the same because of their similar pKa values. The presence of more thiol group content in CMC-Cys in comparison with CMC-Cysteamine is the major reason for its slow enzymatic degradation.

CONCLUSIONS

Within this study, biodegradable thiomers and cross-linked thiomers have been synthesized and examined for enzymatic degradation. Despite the chemical modification, they are still biodegradable and show generally a slower degradation in comparison to unmodified polymers. The degradation rate was found to be strongly dependent on the content of thiol groups, pH value of the reaction medium, structure of the conjugates, the cross-linking of thiomers and the molecular weight of conjugates. In the light of these results, the successful development of a mucoadhesive delivery system based on thiolated polymer requires the recognition of the biodegradation process and its influence on drug release. According to these results, the thiomers would be useful and successful tools for long-term drug delivery applications because they degrade in the body to biologically inert and compatible molecules. Furthermore, thiomers with suitable biodegradation rate could be highly advantageous especially when designing a pulmonary drug delivery system. A new challenging field of thiomer application would be as scaffold materials in cell therapy and tissue engineering.

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